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A Comprehensive *Drosophila melanogaster* Transcription Factor Interactome

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Abstract: Combinatorial interactions among transcription factors (TFs) play essential roles in generating gene expression specificity and diversity in metazoans. Using yeast 2-hybrid (Y2H) assays on nearly all sequence-specific *Drosophila* TFs, we identified 1,983 protein-protein interactions (PPIs), more than doubling the number of currently known PPIs among *Drosophila* TFs. For quality assessment, we validated a subset of our interactions using MITOMI and bimolecular fluorescence complementation assays. We combined our interactome with prior PPI data to generate an integrated *Drosophila* TF-TF binary interaction network. Our analysis of ChIP-seq data, integrating PPI and gene expression information, uncovered different modes by which interacting TFs are recruited to DNA. We further demonstrate the utility of our *Drosophila* interactome in shedding light on human TF-TF interactions. This study reveals how TFs interact to bind regulatory elements *in vivo* and serves as a resource of *Drosophila* TF-TF binary PPIs for understanding tissue-specific gene regulation.

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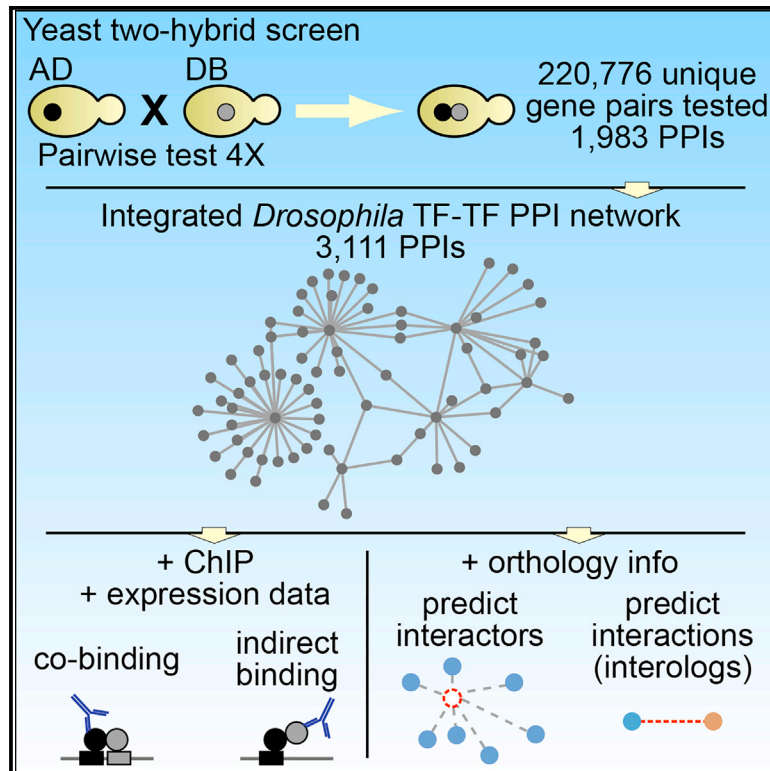
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A Comprehensive *Drosophila melanogaster* Transcription Factor Interactome

Graphical Abstract



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In Brief

Combinatorial regulation by transcription factors (TFs) is one mechanism for achieving condition and tissue-specific gene regulation. Shokri et al. mapped TF-TF interactions between most *Drosophila* TFs, reporting a comprehensive TF-TF network integrated with previously known interactions. They used this network to discern distinct TF-DNA binding modes.

Highlights

- Comprehensive yeast PPI screen among essentially all *D. melanogaster* TFs
- A subset of Y2H results validated by *in vitro* and *in vivo* binary interaction assays
- Compilation of an integrated fly TF-TF interactome
- TF pairs can be recruited to DNA in different modes



A Comprehensive *Drosophila melanogaster* Transcription Factor Interactome

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SUMMARY

Combinatorial interactions among transcription factors (TFs) play essential roles in generating gene expression specificity and diversity in metazoans. Using yeast 2-hybrid (Y2H) assays on nearly all sequence-specific *Drosophila* TFs, we identified 1,983 protein-protein interactions (PPIs), more than doubling the number of currently known PPIs among *Drosophila* TFs. For quality assessment, we validated a subset of our interactions using MITOMI and bimolecular fluorescence complementation assays. We combined our interactome with prior PPI data to generate an integrated *Drosophila* TF-TF binary interaction network. Our analysis of ChIP-seq data, integrating PPI and gene expression information, uncovered different modes by which interacting TFs are recruited to DNA. We further demonstrate the utility of our *Drosophila* interactome in shedding light on human TF-TF interactions. This study reveals how TFs interact to bind regulatory elements *in vivo* and serves as a resource of *Drosophila* TF-TF binary PPIs for understanding tissue-specific gene regulation.

INTRODUCTION

Transcription factors (TFs) are essential for the regulation of gene expression during development and in response to environmental perturbations. One mechanism for achieving gene expression specificity and diversity in metazoans is the binding of multiple TFs at *cis*-regulatory elements. Such combinatorial interactions provide a means to integrate information about cell identity, cell state, and extracellular signals into condition-specific transcriptional responses and are essential in specifying tissue-specific programs during development (Carroll et al.,

2005; Levine and Tjian, 2003; Michelson and Bulyk, 2006; Spitz and Furlong, 2012). Mapping the network of binary protein-protein interactions (PPIs) among TFs is therefore critical for understanding the regulatory interactions through which the specificity of gene expression programs is determined.

Several genome-scale PPI studies have focused on TFs. For humans and mice, a mammalian 2-hybrid approach was used to screen approximately half of an established catalog of human and mouse TFs, identifying 762 and 877 high-stringency interactions, respectively (Ravasi et al., 2010). For *Caenorhabditis elegans*, a yeast 2-hybrid (Y2H) approach assayed PPIs among 834 (89%) TFs and detected 2,253 high-confidence interactions among 437 TFs (Reece-Hoyes et al., 2013). A study using affinity chromatography followed by mass spectrometry (AP-MS) assayed 647 *Drosophila melanogaster* proteins, 229 of which were sequence-specific TFs, in the S2R+ cell line; 624 high-confidence interactions were identified, with 9.5% of interactions constituting binary TF-TF interactions (Rhee et al., 2014). Other studies assayed only small subsets of TFs (Grigoryan et al., 2009; Grove et al., 2009), detected relatively few TF-TF interactions as part of a larger PPI screen (Formstecher et al., 2005; Giot et al., 2003; Stanyon et al., 2004), or inferred TF PPIs from co-expression data and require independent experimental support (Adryan and Teichmann, 2010). In total, prior studies have identified 1,161 interactions among 468 *Drosophila* TFs (Friedman et al., 2011) (Table 1). Considering that there are 755 predicted *Drosophila* TFs (Hens et al., 2011), a large fraction of the *Drosophila* binary TF interactome ("TF-TF interactome") remains to be mapped.

D. melanogaster serves as a powerful metazoan model organism for studies of transcriptional regulation because of its complexity in spatiotemporal gene regulation and abundance of available genetic and genomic resources (Beckingham et al., 2005). Gene expression profiling has been performed across a wide range of developmental time points (Chintapalli et al., 2007; Graveley et al., 2011; Hammonds et al., 2013), and genomic occupancies of numerous TFs have been profiled by



Table 1. Experimentally Identified *Drosophila* TF-TF PPIs

Study	Method	Total no. PPIs Detected	No. of TF-TF Interactions ^a
Giot et al., 2003	Y2H	20,405	341
Stanyon et al., 2004	Y2H	1,814	19
Formstecher et al., 2005	Y2H	2,338	53
Guruharsha et al., 2011	AP-MS	209,912	221
Lowe et al., 2014	AP-MS	14,932	21
Rhee et al., 2014	AP-MS	174,561	23
MasterNet (PPIs detected for <i>Drosophila</i> proteins)	compilation	–	1,161
MasterNet (including PPIs inferred from Interologs)	compilation	–	4,606
This study	Y2H	–	1,983

^aNumbers retrieved from MasterNet.

chromatin immunoprecipitation (ChIP) using DNA microarrays or sequencing (Iyer et al., 2001; Johnson et al., 2007; Lieb et al., 2001; Ren et al., 2000; Wei et al., 2006). The highly evolutionarily conserved nature of many *Drosophila* protein sequences and their regulatory roles (Shubin et al., 2009) makes *Drosophila* a compelling system in which a comprehensive TF-TF interactome would broadly facilitate studies of tissue-specific transcriptional regulation.

Here, we performed a high-throughput Y2H screen to map binary interactions between essentially all full-length *Drosophila* TFs. Y2H provides a more condition-independent survey of PPIs in a nuclear environment than does AP-MS and is not biased toward high-affinity, stable interactions. Furthermore, our approach individually tested binary TF pairs, in contrast to pooled approaches used by other Y2H screens, and offers the advantage of being able to distinguish between negative (i.e., no interaction) and untested interactions. Our Y2H screen resulted in 1,983 unique TF-TF interactions involving 584 TFs, representing a 168% increase in the number of known PPIs among *Drosophila* TFs. We found that the motifs of interacting TFs co-occur within ChIP followed by microarray hybridization and high-throughput sequencing (ChIP-chip/seq) peaks. We further integrated available ChIP-chip/seq and gene expression data to dissect the DNA recruitment mechanisms of interacting TF pairs and built a catalog of direct and indirect TF-DNA interactions in *Drosophila* development. Finally, we demonstrate how this *Drosophila* TF-TF interactome can be used to elucidate gene regulatory networks in humans.

RESULTS

A Binary Interaction Screen among Nearly All *Drosophila* TFs

We compiled a list of 755 predicted sequence-specific TFs in *D. melanogaster* using information from a prior cataloging of

sequence-specific TFs for the FlyTF database (Adryan and Teichmann, 2006), as well as manual curation (Hens et al., 2011). For 720 (95%) of these TFs, Gateway-compatible Entry clones were available (Hens et al., 2011), and we generated Y2H-compatible AD (“prey”) and DB (“bait”) expression vectors, resulting in 695 and 575 successful clones, respectively (Table S1). We tested and analyzed a total of 385,431 AD-DB pairs in quadruplicate using our high-throughput Y2H assay (Walhout and Vidal, 2001) (see STAR Methods) (Figure 1A). These interactions corresponded to 220,776 unique TF pairs (Table S1). We detected 1,983 unique interactions between 584 TFs (Figure 1B; Table S2), including 26 putative homodimeric interactions. Of these interactions, 1,950 (98%) had not been previously identified experimentally.

The frequency of interactions we detect here (1,983/220,776 = 0.898%) is much higher than that obtained in other Y2H screens in flies (~0.004%, Giot et al., 2003), *C. elegans* (~0.002%, Simonis et al., 2009), or humans (~0.002%, Rual et al., 2005; ~0.01%, Stelzl et al., 2005; or 0.15%, Rolland et al., 2014). Since those prior screens were performed on a wide range of genes not limited to TFs, our higher PPI rate may reflect a greater tendency of TFs to interact with one another, stemming from their often-combinatorial control of gene regulation. Among the 584 TFs represented in our interactome, the median number of interactions per TF was 4, and the network density was 0.011. These figures are comparable to those of TF-TF PPIs in other species (Ravasi et al., 2010; Reece-Hoyes et al., 2013). This network follows a scale-free degree distribution (power law fit with $R^2 = 0.81$).

Based on the distribution of the numbers of interacting partners for each TF (Figure 1C), we chose 50 interactors as a threshold for identifying “hub” proteins that are particularly well connected in our Y2H network. Hub proteins are more likely to participate in a higher number of cellular processes and exhibit pleiotropy (Yu et al., 2008). Nine TFs met this criterion (Figure 1C): CG10654, Eip78C, Foxo, Drm, Irbp18, CG31955, Ets65A, Rel, and Hr78. The orthologs of these hub proteins are also highly enriched for being highly connected nodes in *C. elegans* and human TF-TF interaction networks ($p < 2.2 \times 10^{-16}$, Wilcoxon rank sum test) (Figure S1) (Ravasi et al., 2010; Reece-Hoyes et al., 2013). This finding supports the conserved, regulatory importance of these TFs in TF-TF networks. For example, Hr78 is an essential nuclear receptor that is thought to function at the top of the ecdysteroid regulatory hierarchy, with null mutations leading to lethality during the third-instar larval stage (Fisk and Thummel, 1998). Irbp18 was recently identified as a critical gene for general DNA double-stranded break repair (Francis et al., 2016). The TFs interacting with Irbp18 in our Y2H network are enriched for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways relevant to DNA repair such as purine and pyrimidine metabolism and base excision repair (Fisher’s exact test, Benjamini-Hochberg adjusted $p = 0.0122$). We did not find enriched KEGG pathways or Gene Ontology (GO) annotation terms for TFs interacting with the other hub TFs, which may be due in part to the incomplete functional characterization of genes. Three of the hub proteins (Ets65A, CG10654, CG31955) are poorly characterized in *Drosophila*, but the key roles of their orthologs shed light on the possible

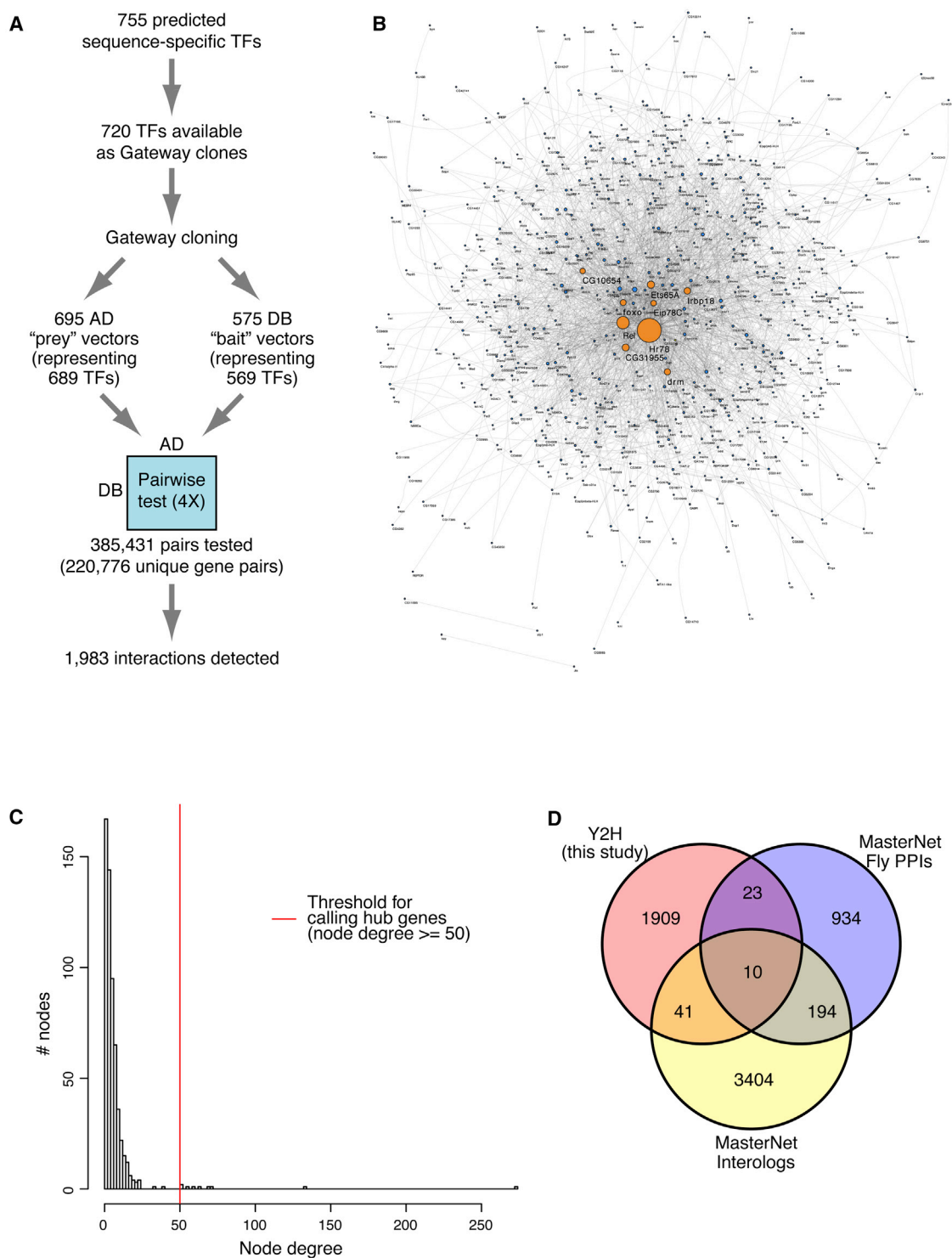


Figure 1. Comprehensive *Drosophila* Y2H TF-TF Interactome

(A) Overview of the Y2H screen.

(B) Network view of the Y2H TF-TF interactome.

(C) Degree distribution of the Y2H interactome. Median degree, 4; mean degree, 6.79. The red vertical line indicates the threshold for calling hub genes (degree, 50).

(D) Comparisons of identified interactions with those in MasterNet.

See also [Figure S1](#) and [Tables S1](#) and [S2](#).

importance of these genes; for example, the Ets65A ortholog Fli1 is one of the master regulators in blood and endothelial development in mouse and zebrafish (Kanki et al., 2017; Liu et al., 2008).

TF-TF Interactome Quality Assessment

As an initial quality assessment of our TF-TF interactome data, we mined the MasterNet database (Friedman et al., 2011), which is a compilation of experimentally determined *Drosophila* PPIs and predicted PPIs based on interactions detected in other species (interologs). We found support for 74 of our 1,983 Y2H PPIs (~4%): 33 had been previously determined experimentally ("MasterNet Fly PPIs"), and 51 were predicted interactions ("MasterNet Interologs") (Figure 1D).

To assess the sensitivity and specificity of our Y2H assay, we assembled reference sets of positive and negative interaction pairs (see STAR Methods). To our knowledge, prior *Drosophila* PPI screens did not use such reference sets to evaluate the quality of their derived interaction data. Our positive reference set (PRS) represents a high-confidence set of 41 *Drosophila* TF-TF interactions supported by at least two pieces of evidence in the literature (Table S3). As it is not possible to obtain a high-confidence set of non-interacting pairs (i.e., true-negative interactions), we generated 1,000 random reference sets (RRSs) (Venkatesan et al., 2009) as proxies for non-interacting TFs. Each RRS comprised a set of 1,983 TF pairs that have not been described as interacting. Our Y2H data recovered 7 interactions in the PRS, yielding a sensitivity of 17% with a false-positive rate (here, the fraction of RRS pairs scoring positive) of 1.2%. These rates are comparable to those of two prior human Y2H interactomes (Rual et al., 2005; Venkatesan et al., 2009), suggesting that our *Drosophila* TF-TF interactome map is of similar quality.

Next, we experimentally validated the quality of our Y2H data using two orthogonal binary interaction assays: an *in vitro* mechanically induced trapping of molecular interactions (MITOMI) assay adapted to identify PPIs (Gerber et al., 2009) and an *in vivo* bimolecular fluorescence complementation (BiFC) assay (Remy and Michnick, 2004) (Figure 2). Briefly, in MITOMI, TF pairs are expressed *in vitro* either as enhanced GFP (eGFP) or mCherry fusions (Isakova et al., 2016) and introduced into the MITOMI chip; TFs are defined as interacting if, after trapping in the MITOMI chip, the protein trapping area is positive for both eGFP and mCherry signal. In BiFC, two non-fluorescent fragments of yellow fluorescent protein (YFP) are fused to two proteins being tested for their ability to interact; if the two proteins interact, then YFP is reconstituted, and a detectable YFP fluorescence signal is emitted (Hu et al., 2002; Pusch et al., 2011).

We used MITOMI or BiFC to test PPIs among TF pairs that are (1) scored positive in our Y2H screen ("Y2H-positive pairs") and not members of the PRS, (2) members of the PRS, or (3) scrambled pairings of TFs in the PRS (Figure 2; Table S4). Of 73 Y2H-positive pairs tested, 39 (53.4%) were positive by either MITOMI or BiFC (representative examples shown in Figures 2B and 2C; examples of different signal intensities shown in Figure S2). Of 12 tested TF pairs from the scrambled PRS pairings, none were positive by either MITOMI or BiFC, supporting the specificity of these assays. In comparison, 9 of 12 probed TF pairs in the PRS tested positive by either MITOMI or BiFC; notably,

TF pairs in the PRS that were positive in our Y2H screen had a higher validation rate (6/7) than those that were negative in our Y2H screen (3/5). These validation rates, together with the sensitivity and specificity determined above, suggest that our Y2H PPI data are of high quality.

Assembly of an Integrated *Drosophila* TF-TF PPI Network

We integrated our Y2H results with data for 1,161 TF-TF interactions that we extracted from MasterNet (Friedman et al., 2011) to assemble a comprehensive network of known *Drosophila* TF-TF PPIs. While acknowledging that MasterNet-derived interactions may also include higher-order interactions, our integrated *Drosophila* TF-TF interactome ("integrated network"; Table S2) comprises 3,111 TF-TF PPIs, 1,950 of which were newly contributed by our Y2H data. This addition represents a 168% increase in the number of experimentally determined fly TF-TF PPIs.

Using this integrated network, we examined which TF families interact with one another (Table S2). Just over 3% (98/3,111) of interactions are homodimeric interactions. Many of these interactions are within basic leucine zipper (bZIP) TFs and helix-loop-helix (HLH) TFs, each of which are known to form homo- and heterodimers. C2H2 zinc finger (ZF) TFs represent the largest group of homodimerizing TFs (20/98) and intrafamily heterodimerizing TFs (218/381) in the integrated network. We found that heterodimeric interactions occur primarily between different TF families (2,356/3,013); this figure is a conservative estimate due to the exclusion of 276 TF-TF interactions in which both TFs were classified as "other" for TF family membership. C2H2 ZF-homeodomain TFs are the most common type of interfamily heterodimers (161/3,013), followed by C4 ZF-C2H2 ZF heterodimers (127/3,013). The large number of ZF TFs represented in these TF-TF interactions is consistent not only with the large number of ZFs represented in the integrated network but also with the known ability of ZFs to mediate PPIs (Brayer and Segal, 2008).

Utility of the TF-TF Interactome Network in the Analysis of *cis*-Regulatory Regions

To investigate whether Y2H-detected TF pairs may co-regulate genes *in vivo*, we investigated ChIP-chip/seq data on TF occupancies (Jakobsen et al., 2007; MacArthur et al., 2009; modENCODE Consortium et al., 2010). Interacting TFs can bind DNA through multiple different modes. In some cases, both partners bind to their cognate DNA binding site motif, and the extent of TF-TF cooperativity is influenced by the composition of the respective heterodimeric binding site (Isakova et al., 2016). In other cases, only one partner binds DNA directly and the other partner is recruited to DNA through PPIs. Such direct versus indirect DNA binding can occur in a cell-type- or condition-specific manner (Gordán et al., 2009, 2011; Mariani et al., 2017). To investigate whether the PPIs that we detected *in vitro* also exist *in vivo*, we inspected genomic regions occupied by a TF for the DNA binding site motif of itself and its partner TF in tissues in which the TFs are co-expressed.

Such an analysis of TF occupancy is dependent on the availability of high-quality TF DNA binding site motif data. To supplement publicly available TF binding motifs in FlyFactorSurvey (Zhu et al., 2011), we generated high-quality motifs for 44 TFs,

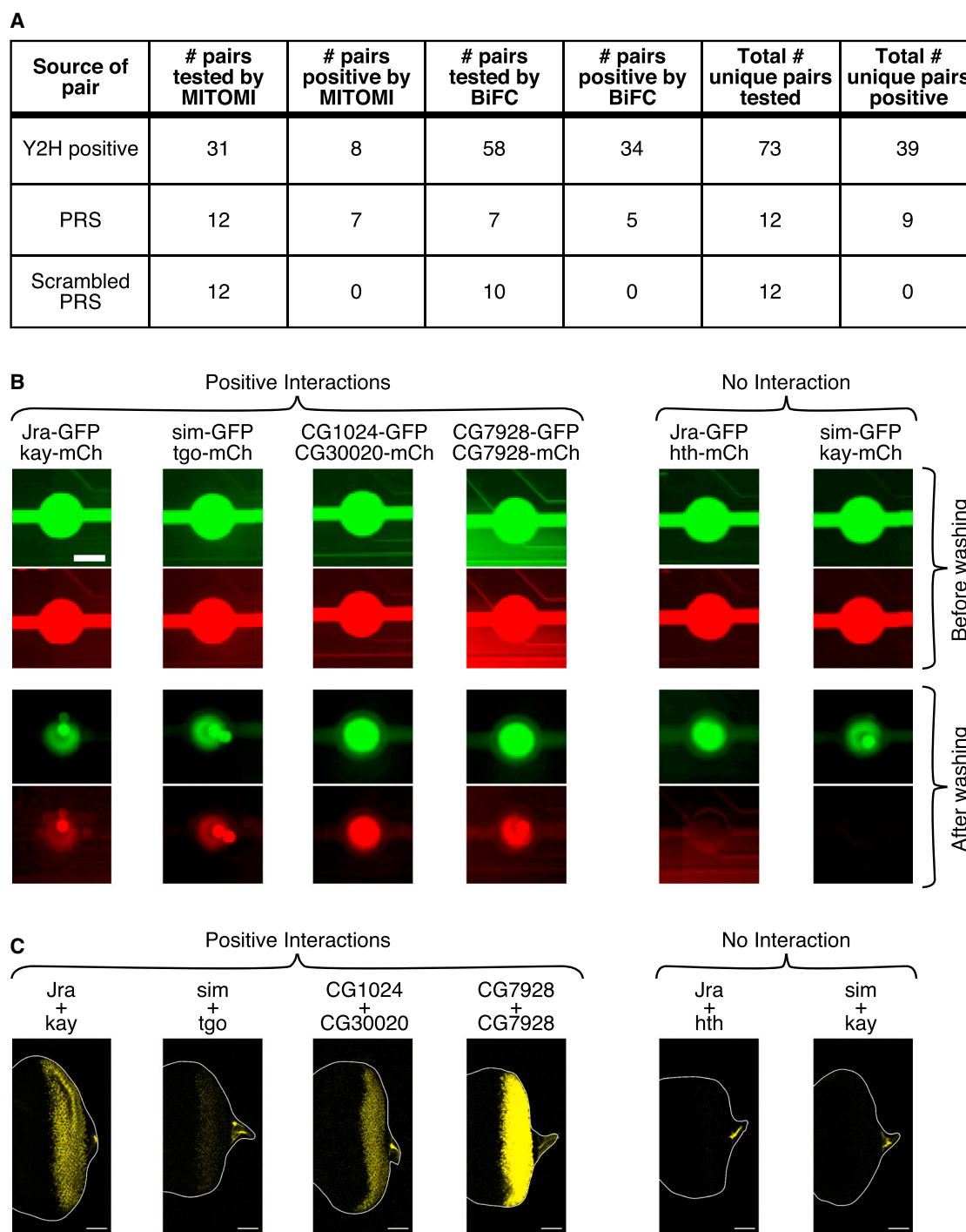


Figure 2. Orthogonal Assays Validate Y2H Interactions

Summary of Y2H interactions validated by MITOMI and BiFC (A). Representative examples of positive and negative interactions assayed by (B) MITOMI and (C) BiFC. The scale bars in (B) are 250 μ m; the scale bars in (C) are 50 μ m. The depicted BiFC interactions (C) were tested in *Drosophila* eye antennal discs.

See also Figure S2 and Tables S3 and S4.

including 9 TFs lacking prior motif data, by universal protein binding microarray (PBM) assays (Berger et al., 2006) (Table S5). In total, we compiled 307 motifs representing 304 TFs (Table S6) (see STAR Methods).

For the TF occupancy analysis, we filtered the integrated network to retain 333 TF pairs for which (1) the TF pair exhibits overlapping expression patterns, (2) DNA binding specificity data are available for both TFs, (3) ChIP-chip/seq data are

available for at least one of the interacting TFs, (4) the expression patterns of the TFs overlapped with the time point(s) of the ChIP-chip/seq data, and (5) the DNA binding specificity motifs of the interacting TFs were dissimilar (using Pearson correlation coefficient <0.8 as a threshold, as in Kheradpour et al., 2007), to distinguish which TF motif was enriched. In all, we considered 57 ChIP-chip/seq datasets that represent these 333 TF pairs (Bradley et al., 2010; Harrison et al., 2011; Jakobsen et al., 2007; MacArthur et al., 2009; Meireles-Filho et al., 2014; modENCODE Consortium et al., 2010; Nevil et al., 2017; Paris et al., 2013; Ren et al., 2015; Shlyueva et al., 2014; Zinzen et al., 2009; Zolotarev et al., 2016). For each of these filtered TF pairs, we evaluated the enrichment of the binding motif of each TF within the ChIP “bound” regions as compared to background genomic regions (Tables 2 and S7) (Wilcoxon rank sum test, Benjamini-Hochberg false discovery rate $q < 0.1$) (Gordán et al., 2009; Mariani et al., 2017) (see STAR Methods).

We found 16 unique TF co-binding pairs (10 from our Y2H interactions, 6 from previously known interactions), in which the motif of the ChIP-profiled TF was enriched along with the motif of at least one of its interacting partner TFs. Our analysis recovered well-known examples of interacting TFs. For example, in Hth (homothorax) ChIP-seq data obtained from stage E0–8 embryos (modENCODE Consortium et al., 2010), we observed enrichment of the Hth motif together with the Exd (extradenticle) and Ubx (ultrabithorax) motifs. Hox proteins such as Ubx are known to interact with Exd and Hth and bind DNA cooperatively *in vivo* as part of a trimeric complex (Ryoo et al., 1999). Interactions between Hth, Exd, and Ubx had been observed in a prior Hth ChIP-chip study of leg and haltere imaginal discs (Slattery et al., 2011).

In another co-binding interaction, we found the cycle (Cyc) motif to be co-enriched with the Foxo motif (Figures 3A and 3B). Cyc forms a heterodimer with clock (Clk) in circadian rhythm, and together activate key circadian rhythm genes (Al-lada et al., 2001). Foxo is the effector TF of the insulin signaling pathway (IIS), which is implicated in longevity and stress response (Tatar et al., 2003). While Cyc and Foxo were not known previously to physically interact, evidence for their overlapping function exists in the literature. For example, under oxidative stress, Clk activity is repressed in cultured *Drosophila* cells, and foxo mutant flies become arrhythmic (Zheng et al., 2007). We observed Cyc and Foxo co-binding in the promoter region of *vrille* (Figure 3B), which has been shown to be a transcriptional target of Cyc-Clk in circadian rhythm (Cyran et al., 2003; Glossop et al., 2003) and of Foxo in the context of lifespan extension (Bai et al., 2013). Thus, it is possible that Foxo and Cyc link IIS and circadian rhythm. Motif co-enrichment for physically interacting, co-binding TFs was also observed in human Encyclopedia of DNA Elements (ENCODE) ChIP-seq data using a similar analysis approach (Mariani et al., 2017).

In 24 cases, the motif of the ChIP-profiled TF was not enriched, but instead the motif of at least one of its interacting TFs was enriched (21 from Y2H, 3 from previously known interactions). This finding suggests that, in those cellular conditions, the profiled factor associates with DNA indirectly through a recruiting TF. Such indirect binding was observed previously in analyses of

Saccharomyces cerevisiae ChIP-chip (Gordán et al., 2009) and human ENCODE ChIP-seq datasets (Mariani et al., 2017; Wang et al., 2012). For most ChIP-profiled TFs in this category, we detected only 1–2 partner TF motifs. However, in the case of Hr78, 14 partner TF motifs were enriched, which is consistent with our finding that Hr78 is a hub protein in our Y2H network. Hr78 was shown to interact with >100 ecdysteroid-regulated chromosomal puff loci (Fisk and Thummel, 1998). One of the TFs that, based on our analyses, may recruit Hr78 is Hairless (Figure 3C), which is induced following steroid hormone 20-hydroxyecdysone (20-HE) treatment of cultured larval organs (Beckstead et al., 2005) and plays a role in 20-HE-regulated cellular differentiation (Gauhar et al., 2009). While the other Hr78 interaction partners are not known to function in ecdysteroid signaling, our results suggest that partner TF-mediated recruitment of Hr78 to genomic loci could direct the transcriptional regulatory role of Hr78 in ecdysteroid signaling. For example, we observed the Hairless motif overlapping an Hr78 ChIP peak upstream of *kayak*, a putative transcriptional target of Hairless (Bianchi-Frias et al., 2004) (Figure 3D); this sequence falls within a chromosomal region previously identified as a 20-HE-regulated chromosomal puff (Fisk and Thummel, 1998).

Of the remaining TF-TF pairs, we observed the enrichment of only the ChIP-profiled TF for 113 pairs, and no enrichment of the ChIP-profiled or partner TF for 180 pairs. The lack of enrichment may be due to the profiled TF interacting with a factor that is absent from our TF interactome, incomplete motif models, or noise in the ChIP-chip/seq data. Alternatively, the profiled TF may bind DNA indirectly through interaction with a variety of different TFs, each of which recruits the profiled TF to only a small fraction of the regions occupied *in vivo*.

Analysis of *Drosophila* TF-TF Interactome Identifies Potential Human Disease TFs

Our integrated *Drosophila* TF PPI network can provide useful insights into human TF-TF interactions. We asked which new TFs and TF-TF interactions may be relevant for human congenital heart disease (CHD), as TFs involved in CHD have been studied extensively (McCulley and Black, 2012). There are 19 human TFs annotated in the Human Gene Mutation Database (HGMD) with the phenotype “congenital heart disease.” We identified fly orthologs of these TFs along with all of their immediately interacting TFs (“first neighbors”) in our fly network. We then restricted the selection to those TFs expressed in the *Drosophila* heart to select heart-relevant PPIs. The resulting network, composed of 28 nodes and 54 edges, is shown in Figure 4A. While we had started out with 12 CHD-associated TF fly orthologs, our approach identified 11 additional TFs that may play important roles in CHD based on interactions with known CHD-associated TFs (blue nodes in connected component). From a literature search, we found that human orthologs of 4 of these 11 fly TFs are important for normal development of the heart. We also found 4 fly TFs that are crucial for normal fly heart development, but their human orthologs are not well studied. The remaining three genes have not yet been implicated in CHD or heart development in any species. Thus, these TFs may be good candidates for future study into their potential roles in human heart development and disease.

Table 2. Evidence of Interacting TFs Found in ChIP Data

Binding Mode	PPI	TF1 (ChIP-Profiled TF)	Adjusted p	TF2	Adjusted p	Overlapping Expression Patterns	ChIP Data Ref.	Time Point of ChIP Data
Co-binding	bcd Rel*	bcd	1.01E-40	Rel	9.16E-2	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, adult female	Paris et al., 2013	E2-4
Co-binding	bcd Rel*	bcd	2.47E-7	Rel	4.72E-2	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, adult female	MacArthur et al., 2009	E2-3
Co-binding	bcd ttk	bcd	2.47E-7	ttk	4.29E-2	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, adult female	MacArthur et al., 2009	E2-3
Co-binding	cyc foxo*	cyc	1.74E-15	foxo	1.86E-4	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	Meireles-Filho et al., 2014	adult female
Co-binding	cyc foxo*	cyc	4.07E-12	foxo	3.44E-5	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	Meireles-Filho et al., 2014	adult female
Co-binding	D ttk	D	4.99E-5	ttk	6.78E-2	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	E1-12
Co-binding	da caup*	da	2.43E-2	caup	2.43E-2	E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	MacArthur et al., 2009	E2-3
Co-binding	da foxo*	da	2.43E-2	foxo	6.23E-2	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	MacArthur et al., 2009	E2-3
Co-binding	da twi	da	2.43E-2	twi	9.22E-2	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	MacArthur et al., 2009	E2-3
Co-binding	hth exd*	hth	1.81E-3	exd	1.86E-4	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	E0-8
Co-binding	hth Ubx	hth	1.81E-3	Ubx	1.47E-4	E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	E0-8
Co-binding	Kr Hr78*	Kr	2.96E-45	Hr78	8.30E-2	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae	Paris et al., 2013	E2-4
Co-binding	Kr Hr78*	Kr	4.16E-60	Hr78	4.98E-4	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae	Paris et al., 2013	E2-4

(Continued on next page)

Table 2. Continued

Binding Mode	PPI	TF1 (ChIP-Profiled TF)	Adjusted p	TF2	Adjusted p	Overlapping Expression Patterns	ChIP Data Ref.	Time Point of ChIP Data
Co-binding	Kr ovo	Kr	4.16E-60	ovo	6.44E-2	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae	Paris et al., 2013	E2-4
Co-binding	Kr Ptx1*	Kr	4.16E-60	Ptx1	6.08E-4	E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae	Paris et al., 2013	E2-4
Co-binding	Trl foxo*	Trl	3.93E-24	foxo	4.09E-2	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	E8-16
Co-binding	Trl ken	Trl	3.93E-24	ken	6.23E-2	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	E8-16
Co-binding	zld Kah*	zld	1.88E-118	Kah	4.79E-46	E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	Harrison et al., 2011	E2-2.5
Co-binding	zld Kah*	zld	9.75E-123	Kah	1.85E-41	E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	Harrison et al., 2011	E3-3.5
Co-binding	zld mirr*	zld	1.88E-118	mirr	6.23E-2	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	Harrison et al., 2011	E2-2.5
Co-binding	zld mirr*	zld	9.75E-123	mirr	4.09E-2	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	Harrison et al., 2011	E3-3.5
Indirect	br Zif*	br	1.00E+00	Zif	9.81E-2	E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	WPP
Indirect	Dll CG3919*	Dll	1.00E+00	CG3919	3.63E-2	E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	WPP
Indirect	Dll mirr*	Dll	1.00E+00	mirr	3.63E-2	E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	WPP
Indirect	Dll rn*	Dll	1.00E+00	rn	2.07E-7	E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	WPP
Indirect	EcR Rel*	EcR	8.34E-1	Rel	6.15E-2	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	Shlyueva et al., 2014	WPP

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Table 2. Continued

Binding Mode	PPI	TF1 (ChIP-Profiled TF)	Adjusted p	TF2	Adjusted p	Overlapping Expression Patterns	ChIP Data Ref.	Time Point of ChIP Data
Indirect	gt lola	gt	5.07E−1	lola	6.23E−2	E0–2, E2–4, E4–6, E6–8, E8–10, E10–12, E12–14, E14–16, E16–18, E18–20, E20–22, E22–24, L1, L2, L3, WPP, pupae, adult male, adult female	Paris et al., 2013	E2–4
Indirect	gt lola	gt	7.22E−1	lola	8.89E−2	E0–2, E2–4, E4–6, E6–8, E8–10, E10–12, E12–14, E14–16, E16–18, E18–20, E20–22, E22–24, L1, L2, L3, WPP, pupae, adult male, adult female	Paris et al., 2013	E2–4
Indirect	gt ttk	gt	5.07E−1	ttk	5.65E−3	E0–2, E2–4, E4–6, E6–8, E8–10, E10–12, E12–14, E14–16, E16–18, E18–20, E20–22, E22–24, L1, L2, L3, WPP, pupae, adult male, adult female	Paris et al., 2013	E2–4
Indirect	Hr78 bowl*	Hr78	1.00E+00	bowl	6.81E−2	E0–2, E2–4, E4–6, E6–8, E8–10, E10–12, E12–14, E14–16, E16–18, E18–20, E20–22, E22–24, L1, L2, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	E8–16
Indirect	Hr78 disco*	Hr78	1.00E+00	disco	6.81E−2	E0–2, E2–4, E4–6, E6–8, E8–10, E10–12, E12–14, E14–16, E16–18, E18–20, E20–22, E22–24, L1, L2, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	E8–16
Indirect	Hr78 Doc2*	Hr78	1.00E+00	Doc2	1.47E−4	E0–2, E2–4, E4–6, E6–8, E8–10, E10–12, E12–14, E14–16, E16–18, E18–20, E20–22, E22–24, L1, L2, L3, WPP, pupae	modENCODE Consortium et al., 2010	E8–16
Indirect	Hr78 E (spl)m3-HLH*	Hr78	1.00E+00	E(spl)m3-HLH	4.54E−2	E2–4, E4–6, E6–8, E8–10, E10–12, E12–14, E14–16, E16–18, E18–20, E20–22, E22–24, L1, L2, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	E8–16
Indirect	Hr78 E (spl)mdelta-HLH*	Hr78	1.00E+00	E(spl)mdelta-HLH	9.14E−5	E0–2, E2–4, E4–6, E6–8, E8–10, E10–12, E12–14, E14–16, E16–18, E18–20, E22–24, L3, WPP, pupae	modENCODE Consortium et al., 2010	E8–16
Indirect	Hr78 ey*	Hr78	1.00E+00	ey	6.10E−5	E0–2, E2–4, E4–6, E6–8, E8–10, E10–12, E12–14, E14–16, E16–18, E18–20, E20–22, E22–24, L1, L2, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	E8–16
Indirect	Hr78 Fer3*	Hr78	1.00E+00	Fer3	3.25E−2	E6–8, E8–10, E10–12, E12–14, E14–16, E16–18, E18–20, E20–22, E22–24, L1, L2, L3, pupae, adult male	modENCODE Consortium et al., 2010	E8–16
Indirect	Hr78 h*	Hr78	1.00E+00	h	1.31E−3	E0–2, E2–4, E4–6, E6–8, E8–10, E10–12, E12–14, E14–16, E16–18, E18–20, E20–22, E22–24, L1, L2, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	E8–16
Indirect	Hr78 Max*	Hr78	1.00E+00	Max	4.72E−2	E0–2, E2–4, E4–6, E6–8, E8–10, E10–12, E12–14, E14–16, E16–18, E18–20, E20–22, E22–24, L1, L2, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	E8–16
Indirect	Hr78 Mondo	Hr78	1.00E+00	Mondo	9.16E−2	E0–2, E2–4, E4–6, E6–8, E8–10, E10–12, E12–14, E14–16, E16–18, E18–20, E20–22, E22–24, L1, L2, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	E8–16

(Continued on next page)

Table 2. Continued

Binding Mode	PPI	TF1 (ChIP-Profiled TF)	Adjusted p	TF2	Adjusted p	Overlapping Expression Patterns	ChIP Data Ref.	Time Point of ChIP Data
Indirect	Hr78 sna	Hr78	1.00E+00	sna	1.31E-3	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae	modENCODE Consortium et al., 2010	E8-16
Indirect	Hr78 sv	Hr78	1.00E+00	sv	1.31E-3	E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	E8-16
Indirect	Hr78 Trl	Hr78	1.00E+00	Trl	7.55E-4	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	E8-16
Indirect	Hr78 zfh1	Hr78	1.00E+00	zfh1	3.25E-2	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	E8-16
Indirect	kn Xbp1	kn	1.00E+00	Xbp1	3.43E-2	E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male	modENCODE Consortium et al., 2010	E0-12
Indirect	prd Hr78	prd	1.00E+00	Hr78	6.23E-2	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E22-24, L3, WPP, pupae, adult male	modENCODE Consortium et al., 2010	E0-12
Indirect	Su(H) da	Su(H)	0.999999984	da	4.72E-2	E0-2, E2-4, E4-6, E6-8, E8-10, E10-12, E12-14, E14-16, E16-18, E18-20, E20-22, E22-24, L1, L2, L3, WPP, pupae, adult male, adult female	modENCODE Consortium et al., 2010	E0-8

Some interactions are listed twice because these pairs were found in multiple ChIP samples.*Interactions found in Y2H.

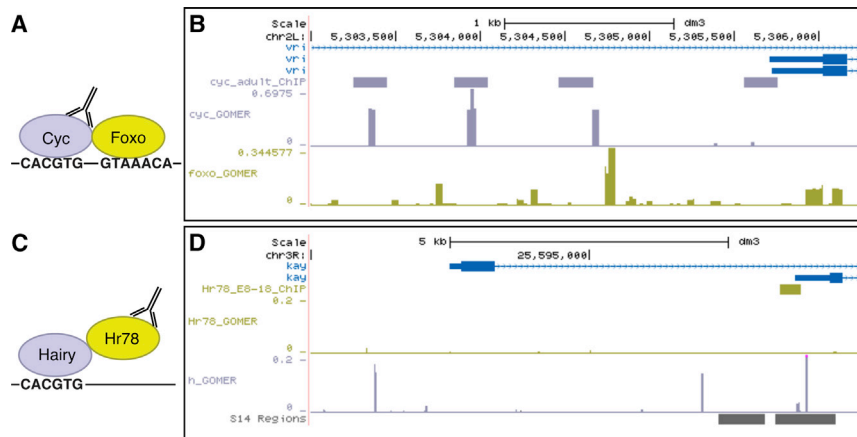


Figure 3. Examples of TF-TF Co-binding and Indirect Binding in Genomic Regions

(A) Schematic representation of Cyc-Foxo co-binding on DNA.

(B) Cyc-Foxo co-binding is observed in the promoter region of *vrille*. GOMER tracks indicate motif enrichment for each TF.

(C) Schematic representation of Hr78 indirect DNA binding through Hair1.

(D) Hr78 indirect binding through Hair1 is observed in the promoter region of *kayak*. GOMER tracks indicate motif enrichment for each TF. “S14 Regions” track indicates open chromatin regions derived from DNase-seq experiments performed at 10–11 h post-hatch (Li et al., 2011) (retrieved from the University of California, Santa Cruz [UCSC] Genome Browser), corresponding to the co-expressed time points of the 2 TFs.

As another example, we examined the *Drosophila* genes implicated in eye development. Although the eye architectures of *Drosophila* and vertebrates are very different, the set of genes that regulate eye development is well conserved (Kumar, 2009). We selected a set of 9 TFs that are key regulators in *Drosophila* eye development (Kumar, 2009) (henceforth, “eye regulators”) and included these genes’ first neighbors, resulting in a network comprising 115 nodes and 128 edges (Figure 4B). Most nodes were connected to only one of the eye regulators, but a handful of genes were connected to multiple eye regulators, potentially indicating their roles in this process. Of note, Hr78 is connected to four eye regulators (So, Eyg, Ey, and Optix). Although Hr78 was previously found to be dispensable for the progression of the morphogenetic furrow in the *Drosophila* eye (Brennan et al., 2001), it may have alternative functions in eye development. Notably, the Hr78 ortholog Nr2c1 has recently been found to regulate early retinal cell patterning in the mouse (Oliveras et al., 2017). Exd interacts with Hth and two eye regulators, Tsh and Ey. Although Exd was not included in the original list of 9 eye regulators, it is known to be a negative regulator of eye development in the fly, functioning together with Hth (Pai et al., 1998). Exd orthologs Pbx2 and Pbx4 regulate eye-patterning genes in zebrafish (French et al., 2007). These findings suggest that other genes that interact with multiple eye regulators similarly may be good candidates for further study for their roles in eye development or function.

DISCUSSION

We report a Y2H-derived *Drosophila* TF-TF interactome representing 77% (584/755) of all *Drosophila* TFs. Our interactome adds 1,950 new interactions to the set of all of the known *Drosophila* TF-TF interactions (Friedman et al., 2011), increasing the total number by 168%. We combine our newly identified interactions with previously known interactions to present the integrated network, which serves as a comprehensive resource of all high-confidence *Drosophila* TF-TF interactions for studies of the TF co-regulation of gene expression (Table S2).

Our integrated *Drosophila* TF-TF network is likely incomplete. Prior studies have noted that there is only limited overlap be-

tween PPIs recovered by different methods, due at least in part to differences in the sensitivities of distinct assay methods that may be inherently better suited for detecting different kinds of biophysical interactions (Braun et al., 2009; Yu et al., 2008). For example, it has been suggested that AP-MS detects stable complexes, whereas Y2H detects more transient or condition-specific PPIs (Yu et al., 2008). Our BiFC experiments suggest that BiFC is a more sensitive assay compared to Y2H, as it is able to detect more PRS interactions.

Y2H sensitivity has been estimated to be ~20%, indicating that the same search space would need to be sampled multiple times to identify all of the biophysical interactions detectable by Y2H (Yu et al., 2008). While we conducted our screen in quadruplicate, additional replicates and the use of alternate Y2H vectors (Stellberger et al., 2010), yeast strains (Braun et al., 2009), or PPI screening technologies will likely improve the coverage of the *Drosophila* TF-TF interactome. Thus, it is important to keep this caveat in mind when interpreting negative Y2H interactions. Furthermore, a broader PPI screen including non-DNA-binding cofactors and chromatin-associated proteins may reveal additional co-regulatory interactions (Babb et al., 2001; Siggers et al., 2011) and higher-order TF complexes that may link transcriptional regulation to signaling pathways and networks.

A TF-TF interaction is not a prerequisite for cooperative DNA binding (Deplancke et al., 2016; Reiter et al., 2017). For example, THRA isoform $\alpha 2$ and RXRA TFs heterodimerize only upon binding to specific DNA sequences (Reginato et al., 1996). Furthermore, DNA-mediated TF-TF cooperativity between TFs that are not known to physically interact with one another may be common (Jolma et al., 2015). Such DNA-mediated or other ligand-dependent TF-TF interactions would not be detected in a conventional Y2H assay.

Interactions between TFs contribute to the spatiotemporal specificity of transcriptional regulation. Accurate models of cell-type- and condition-specific gene regulation will require an improved understanding of how TFs are recruited to DNA, such as via PPIs, and how such interactions affect the DNA binding specificities of the resulting TF complexes. Here, in Table 2, we provide a catalog of co-binding and indirect *Drosophila* TF-DNA interactions inferred from the analysis of ChIP-chip/seq, TF binding motif, gene expression, and PPI data.

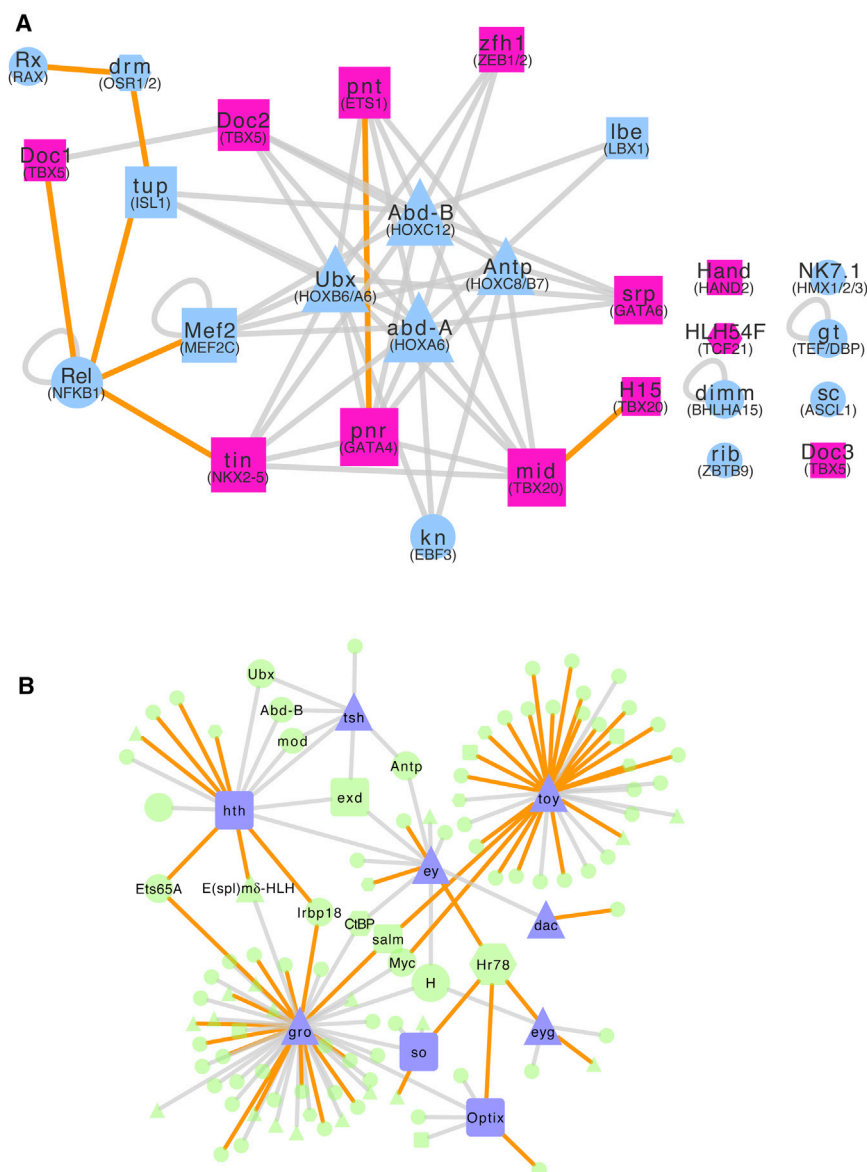


Figure 4. Integrated Fly TF-TF Interactome Identifies Candidate Human TFs Involved in Congenital Heart Disease and Eye Development

(A) The *Drosophila* TF-TF interactome can be used to identify candidate heart development and CHD genes. Nodes: *Drosophila* TFs; edges: PPIs. Nodes with human orthologs that are annotated as CHD associated in HGMD (pink nodes) and their first neighbors (blue nodes) that are expressed in *Drosophila* heart tissue are shown. Unconnected components arose due to this tissue expression requirement. Square: the *Drosophila* gene and its human ortholog(s) are involved in CHD and heart development based on a literature search; triangle: the *Drosophila* gene is involved in heart development; hexagon: the *Drosophila* gene has a human ortholog involved in heart development; circle: neither the *Drosophila* gene nor human ortholog(s) are known to be involved in heart development. Node sizes roughly reflect node degrees. Orange edges indicate newly discovered interactions in this study. Human ortholog gene names are given in parentheses below the *Drosophila* gene name.

(B) The *Drosophila* TF-TF interactome can be used to identify candidate eye development genes. Nodes: *Drosophila* TFs; edges: PPIs. Nodes associated with eye development in *Drosophila* (based on Kumar, 2009; purple nodes) and their first neighbors (green nodes) are shown. Triangle: the *Drosophila* gene has eye development-related GO terms; square: the *Drosophila* gene (shown) and its vertebrate ortholog(s) have eye development-related GO terms; circle: neither the *Drosophila* gene nor vertebrate ortholog(s) have GO terms pertaining to eye development. Orange edges indicate newly discovered interactions in this study. Node sizes roughly reflect node degrees.

Different DNA binding modes of TF partners (i.e., binding to DNA directly versus indirectly via a DNA-bound partner) may reflect condition-specific regulatory mechanisms; we found evidence for both modes of binding in our PPI network. Furthermore, we found that a TF can exhibit multiple DNA binding modes, depending on its interaction partner (Table 2). For example, Hr78 is recruited to DNA by multiple TFs in E8–16, but it recruits Prd to DNA in E0–12. Trl co-binds with Foxo and Ken in E8–16, but it recruits Hr78 at the same developmental stage. Different TF binding modes, including TF monomeric binding, can yield different DNA binding specificities, offering mechanisms by which a TF can regulate unique sets of target genes (Siggers and Gordân, 2014). Further studies are needed to determine whether different modes of TF dimer recruitment to DNA by TF-TF interactions found in our

TF-TF interactome effect distinct transcriptional outputs or are used under different cellular conditions.

TF-TF interactions are important in effecting spatiotemporal-specific transcription. Our systematic TF-TF interactome lays the groundwork for understanding the complexities of combinatorial transcriptional regulation in *Drosophila*. Future investigations are needed to elucidate how these physical interactions translate to dynamic transcriptional control.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.celrep.2019.03.071>.

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AUTHOR CONTRIBUTIONS

B.D. and M.L.B. designed the overall research project and supervised the research. L.S., A.H., K.H., and J.-D.F. performed the Y2H experiments. R.D. performed the MITOMI assays. J.B., E.F., and K.B. performed the BiFC assays. L.S. and A.V. performed the PBM assays. L.S., A.H., S.I., S.S.G., and K.W. performed the computational analyses. L.S., S.I., B.D., and M.L.B. wrote the manuscript.

DECLARATION OF INTERESTS

M.L.B. is a co-inventor on patents on PBM technology.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
GST Tag Polyclonal Antibody, Alexa Fluor 488	ThermoFisher SCIENTIFIC	A-11131; RRID: AB_2534137
Anti-GST polyclonal primary antibody	SIGMA-ALDRICH	G7781; RRID: AB_259965
Anti-HA antibody produced in rabbit	SIGMA-ALDRICH	H6908; RRID: AB_260070
HA Tag Monoclonal Antibody (16B12), Alexa Fluor 488	ThermoFisher SCIENTIFIC	A-21287; RRID: AB_2535829
Monoclonal ANTI-FLAG® M2-Peroxidase (HRP) antibody produced in mouse	SIGMA-ALDRICH	A8592; RRID: AB_439702
FLAG Tag Antibody, Alexa Fluor 488	Cell Signaling TECHNOLOGY	5407; RRID: AB_1950473
Goat HRP-conjugated anti-rabbit IgG	ThermoFisher SCIENTIFIC	31460; RRID: AB_228341
Goat HRP-conjugated anti-mouse IgG	ThermoFisher SCIENTIFIC	32430; RRID: AB_1185566
Anti-GFP antibody (Biotin)	Abcam	ab6658; RRID: AB_305631
Anti-RFP antibody (Biotin)	Abcam	ab34771; RRID: AB_777699
Bacterial and Virus Strains		
<i>E. coli</i> C41 DE3 cells	Lucigen	60444
Gateway Vector Conversion System with One Shot ccdB Survival Cells	ThermoFisher SCIENTIFIC	11828029
Chemicals, Peptides, and Recombinant Proteins		
NeutrAvidin Protein	ThermoFisher SCIENTIFIC	31000
Glutathione S-Transferase from <i>E. coli</i>	SIGMA-ALDRICH	G5663
Amino-terminal FLAG-BAP fusion protein	SIGMA-ALDRICH	P7582
<i>E. coli</i> positive control whole cell lysate	Abcam	Ab5395
Biotinylated BSA	SIGMA-ALDRICH	A8549
Cy3-conjugated dUTP	GE Healthcare	PA53022
Protease, from <i>Streptomyces griseus</i>	SIGMA-ALDRICH	P6911
Thermo sequenase cycle sequencing kit	USB	78500
Gateway® Vector Conversion System with One Shot® ccdB Survival Cells	ThermoFisher SCIENTIFIC	11828029
Carboxy-terminal FLAG-BAP fusion protein	SIGMA-ALDRICH	p7457
Critical Commercial Assays		
PURExpress <i>in vitro</i> transcription and translation kit	NEB	E6800
1-Step Human Coupled IVT Kit - DNA	ThermoFisher SCIENTIFIC	88881
TnT® Coupled Wheat Germ Extract System	Promega	L3260
Deposited Data		
PBM data and derived DNA binding specificity motifs	This paper	http://the_brain.bwh.harvard.edu/uniprobe/ (Accession ID: SHO18A)
TF DNA binding specificity motifs	FlyFactorSurvey (Zhu et al., 2011); CIS-BP (Weirauch et al., 2014)	http://mccb.umassmed.edu/ffs/ ; http://cisbp.ccbr.utoronto.ca/
ChIP datasets	See Table S7	N/A
Y2H TF–TF interactome data	This paper	Table S2
Integrated TF–TF Interactome	This paper	Table S2
MasterNet (Freeze April 2015)	Friedman et al., 2011	N/A
<i>C. elegans</i> TF–TF interactome	Reece-Hoyes et al., 2013	N/A
Human TF–TF interactome	Ravasi et al., 2010	N/A
Human Gene Mutation Database (HGMD)	Stenson et al., 2003	http://www.hgmd.cf.ac.uk/
Berkeley Drosophila Genome Project (BDGP)	Hammonds et al., 2013	http://www.fruitfly.org/

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
<i>D. melanogaster</i> : UAS- <i>TF-3xHA</i> fly strains	Schertel et al., 2015	https://flyorof.ch/
<i>S. cerevisiae</i> : MaV203 and MaV103 strains	Vidal et al., 1996a, 1996b	N/A
Oligonucleotides		
HPLC-purified primer (unmodified) for double-stranding of DNA oligonucleotide array 5'-CAGCACGGACAACGGAACACAGAC-3'	Integrated DNA Technologies	http://www.idtdna.com/pages
Recombinant DNA		
Plasmid: pTSVNm9.attB & pTSVC155.attB	Bischof et al., 2013	https://flyorof.ch/
Plasmid: pT7CFE1-NFtag Vector	ThermoFisher SCIENTIFIC	88865
Plasmid: pT7CFE1-NHA	ThermoFisher SCIENTIFIC	88861
Plasmid: pF3A-eGFP	Isakova et al., 2016	N/A
Plasmid: pF3A-mCherry	Isakova et al., 2016	N/A
Plasmid: pGW-HA.attB	Bischof et al., 2013	https://flyorof.ch/
Plasmid: pAD-DEST	Walhout and Vidal, 2001	N/A
Plasmid: pDB-DEST	Walhout and Vidal, 2001	N/A
Plasmids: <i>Drosophila</i> TF clones collection	Hens et al., 2011	N/A
Software and Algorithms		
Universal PBM Analysis Suite	Berger and Bulyk, 2009	http://the_brain.bwh.harvard.edu/PBMAAnalysisSuite/index.html
MATLAB	N/A	https://www.mathworks.com
TIDY	Hens et al., 2011	Available by request
Python (2.7.12)	N/A	https://www.python.org/
Perl	N/A	https://www.perl.org/
AMIGO2	Balsa-Canto et al., 2016	http://amigo.geneontology.org/amigo/
DRSC Integrative Ortholog Prediction Tool (DIOPT)	Hu et al. 2011	https://www.flyrnai.org/cgi-bin/DRSC_orthologs.pl
liftOver	Hinrichs et al., 2006	https://genome.ucsc.edu/cgi-bin/hgLiftOver
GENRE	Mariani et al., 2017	http://thebrain.bwh.harvard.edu/glossary-GENRE/download.html
GOMER	Granek and Clarke, 2005	http://people.duke.edu/~josh/biophysicsweb/GOMER/index.html
arrayWorx	Applied Precision	(discontinued)
CytoScape (v 3.4.0)	Shannon et al., 2003	https://cytoscape.org
NetworkAnalyzer	Doncheva et al., 2012	Available in CytoScape
ImageJ		https://imagej.nih.gov/ij/
Other		
Custom-designed “universal all 10-mer” oligonucleotide arrays	Agilent Technologies	AMADID #030236

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Martha L. Bulyk. (mlbulyk@genetics.med.harvard.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila strains

For the BiFC analysis, select UAS-*TF-3xHA* fly strains generated earlier (Schertel et al., 2015) were subjected to C-terminal *in vivo* swapping to obtain the *TF-VN* and *TF-VC* strains. These strains contain either the N-terminal Venus YFP fragment VNm9 or the

C-terminal Venus fragment VC155 at the C terminus of the TFs. After the swapping, strains were individually equipped with a *GMR-GAL4* driver (second chromosome) for subsequent expression of the VN- or VC-tagged UAS-transgenes. TF combinations were generated by crossing pairs of *GMR-GAL4;TF-VN* and *GMR-GAL4;TF-VC* strains. All fly progeny from crosses were used without selecting a specific sex. The fly stocks were kept according to general fly husbandry conditions. As described in the “Bimolecular fluorescence complementation assays” section (see [Method Details](#)), BiFC analysis was performed in third instar eye-antennal discs.

Yeast strains

For the Y2H assay, AD (“prey”) and DB (“bait”) clones were transformed into the yeast strains MaV203 and MaV103 ([Vidal et al., 1996a, 1996b](#)), respectively. Growth conditions for Y2H assays are provided under the “Yeast two-hybrid screen” section (see [Method Details](#)).

METHOD DETAILS

Yeast two-hybrid screen

Preparation of TF clones and yeast strain transformation

A list of 755 predicted sequence-specific TFs in *D. melanogaster* was generated from a prior cataloguing of sequence-specific TFs for the FlyTF database ([Adryan and Teichmann, 2006](#)) and through manual curation ([Hens et al., 2011](#)). Our library consisted of 692 clones (of these 755 TFs) obtained from Berkeley *Drosophila* Genome Project (BDGP) cDNA clones (588 were full-length sequence-verified (Gold); 36 clones were end-sequence verified at both 5' and 3' ends (Silver); 68 were partially sequenced (Bronze)), plus five additional TFs that were cloned and full-length sequence-verified for this study. All TF names were updated to the October 2016 release of FlyBase ([Gramates et al., 2017](#)).

The Y2H assay utilizes the product of the yeast gene *GAL4*. The Gal4 TF has two separable domains: the DNA binding domain (DB) and the transcriptional activation domain (AD). Plasmids carrying sequences coding for DB and AD are fused in-frame to DNA sequences coding for proteins of interest and are introduced into yeast strains of opposite mating types. Pairwise combinations of TFs are generated by mating. A physical interaction between the tested proteins reconstitutes the Gal4 protein, resulting in transcription of reporter genes ([Walhout et al., 2000; Walhout and Vidal, 2001](#)).

We cloned our TF library into pAD-DEST and pDB-DEST vectors ([Walhout and Vidal, 2001](#)). We used low copy number yeast expression (ARS/CEN) vectors to control overexpression for low expression to minimize artifactual interactions. We transformed AD (“prey”) and DB (“bait”) clones into the yeast strains MaV203 and MaV103, respectively. A robotic platform automated the yeast transformation process in 384-well plate format ([Hens et al., 2011](#)). Briefly, yeast and TF ORF DNA were mixed using fixed pins and then incubated first at 30°C and then at 42°C. Transformed yeast cells were pelleted using an integrated centrifuge, resuspended, and then spotted on permissive medium. The resulting colonies were then robotically replica-stamped on selective medium (Sc-Trp for AD library, and Sc-Leu for DB library) to select for successful transformants. We successfully cloned and transformed 695 pAD clones and 575 pDB clones, corresponding to 689 and 569 unique TF genes, respectively (some TFs are represented by multiple sequences; see [Table S1](#)). The AD library was then replica-plated in 1536-well format and used for mating. DB-containing strains were individually spread out on plates, from which we robotically replicated 1536 colonies of a single DB strain on yeast extract-peptone-dextrose plates. Mating between the AD and DB strains was achieved by replica-plating the AD library onto the DB colonies on the YEPD plate and incubating overnight at 30°C. Colonies were selected for successful mating on Sc-Leu, -Trp plates. Empty AD vector served as a negative control.

Auto-activation detection

To detect PPIs, the colonies were replica plated on Sc-Leu, -Trp, -His plates containing two different concentrations of 3-aminotriazole (3AT) (20 and 40 mM). 3AT is a competitive inhibitor of His3 and was used to titrate the minimum level of *HIS3* expression required for growth on histidine-deficient medium. Thus, testing colony growth at various 3AT concentrations allowed us to make robust interaction calls using the automated detection protocol described below. Strong, uniform growth of all 1536 colonies on all 3-AT concentrations indicates auto-activation by the DB clone, which can be caused by the presence of an intrinsic transcriptional activation domain in some TFs. 37 pDB-DEST clones were flagged as auto-activating TFs ([Table S1](#)). 19 of these 37 flagged TFs could still be used for Y2H analysis by managing background levels and using our automated interaction detection software.

Omission of pseudogenes from analysis

Two of the 755 predicted TFs corresponded to pseudogenes (FBgn0053221 and FBgn0029920). Though these genes were included in the Y2H assay, they were omitted from further analysis.

Automated detection of interactions in Y2H screen

To process the yeast plate images and detect PPIs in an automated and objective manner, we adapted a MATLAB-based image analysis program, TIDY, which we previously used for the detection of yeast one-hybrid interactions ([Hens et al., 2011](#)). Briefly, the software semi-automatically calls interactions based on pattern detection of yeast colony images convoluted with a 2x2 spot pattern (corresponding to the spot pattern of the quadruplicate colonies), where the size of the spots matches the average size of the colonies. This approach is robust to differing background signals across the same plate. In addition, we computed a homogeneity score over the four replicate spots and penalized positions with 2 strong and 2 weak colonies. TIDY was improved from the original version ([Hens et al., 2011](#)) to provide a Z-score of a given interaction over one or more input images, for example, corresponding to

plates screened at different 3AT concentrations (as described above and used here) or over multiple biological replicate experiments.

To calculate the Z-score, we first calculated the mean and standard deviation of intensities in each image after excluding outlier signals (corresponding to potential interactions) as defined by the Grubbs' test; a normal distribution is expected for the background intensities (position with no interactions). We then normalized intensities across the entire image. As described previously, TIDY offers the option to "correct" for differences in colony growth which are sometimes observed between colonies at the interior versus the exterior regions of the plate. If this option is selected by the user, the normalization is performed independently for the interior and exterior colonies; we used this option as needed, based on visual inspection of the plate. The Z-score represents the average of the normalized intensities for a given interaction over multiple different analyzed images, excluding those images where the colonies are not homogeneous. All images are weighted equally, meaning that an interaction appearing in only one image could be averaged out and not recognized as a positive interaction, if the normalized intensity is not sufficiently strong in the other images.

Due to experimental dropout (e.g., systematic lack of colony growth), we tested and analyzed a total of 385,431 AD/DB pairs corresponding to 220,776 unique TF–TF interactions (Table S1), and detected 1,983 interactions in at least one direction, including 26 self-interactions (i.e., putative homodimers).

Experimental validation of Y2H interactions

MITOMI assays

A subset of our Y2H screen PPIs were tested by an independent experimental methodology using an adaptation of the MITOMI technology (Maerkl and Quake, 2007) to assay for PPIs (Gerber et al., 2009). Briefly, each TF was subcloned from the Entry vector into pF3A-eGFP and pF3A-mCherry vectors and arrayed in duplicate on a slide so that each eGFP-labeled TF was tested against all other TFs and itself as mCherry fusions. TFs were expressed by flowing an IVT reaction mixture over the flow cell. eGFP fusion proteins were trapped by an anti-GFP antibody coated on the slide. PPIs were determined by comparing the mCherry and eGFP signal intensities. We prioritized our Y2H PPIs for testing by MITOMI as follows: TFs resulting in many PPIs ('sticky' proteins), to investigate whether they were true hits or false positives; TF PPIs demonstrating an interaction in only one direction (AD/DB); TF PPIs spanning a wide range of protein domains; TF PPIs involving protein domains not previously known to interact.

Bimolecular fluorescence complementation assays

Briefly, two non-fluorescent fragments of YFP are fused to the ORFs of putatively interacting proteins. If the proteins interact, then YFP is reconstituted and the YFP fluorescent signal can be detected (Hu et al., 2002; Pusch et al., 2011). Selected UAS-TF-3xHA fly strains were subjected to C-terminal *in vivo* swapping to obtain the TF-VN and TF-VC strains, as described in the *Drosophila* strains section above. BiFC analysis of protein–protein interactions was performed in third instar eye-antennal discs. In brief, TF combinations were generated and tested by crossing pairs of GMR-Gal4;TF-VN and GMR-Gal4;TF-VC fly strains, leading to co-expression of the fusion proteins in the eye tissue. The crosses were kept at 21°C until dissection to saturate protein expression levels, which is a critical parameter for maximizing the specificity of BiFC. Eye-antennal discs were dissected from 5–6 third instar larvae and monitored for Venus YFP fluorescence signal with a Zeiss Lsm710 confocal microscope. (For further details, see (Bischof et al., 2013).) The BiFC signals were measured at identical microscope settings, and classified into signal intensity call categories based on visual assessment; for display, all images in Figure 2C and Figure S2 were identically brightness-adjusted (+150) in Adobe Photoshop. Examples of signal intensity calls are shown in Figure S2. "(+)" was not considered to be a positive interaction.

BiFC assays are expected to detect all PPIs in close proximity, including those that are mediated by a third protein. Thus, two TFs that are co-expressed in a given tissue might be more likely to be detected as a positive hit in a BiFC assay conducted in that tissue than two TFs that are not co-expressed. To explore this issue, we examined an RNA-seq dataset from third instar eye-antennal discs (Potier et al., 2014). This stage and tissue correspond to those used in our BiFC experiments. Of the 75 TF–TF pairs tested by BiFC, 48 pairs were co-expressed in the eye-antennal disc. The proportions of positive interactions in the co-expressed versus the non-co-expressed pairs were similar: 25/48 (52.1%) in co-expressed pairs, and 14/27 (51.9%) in the non-co-expressed pairs. Thus, we conclude that the likelihood of scoring a TF–TF positive interaction by BiFC is no higher among TFs that are normally co-expressed in third instar eye-antennal discs than TFs that are not co-expressed.

Computational validation of Y2H results

Comparison of Y2H results to MasterNet

We compared our Y2H results to published PPI data assembled in the MasterNet database (Friedman et al., 2011). Briefly, MasterNet is a compilation of databases, as follows: (i) fly binary PPI network constructed by integrating experimentally identified interactions from major PPI databases (e.g., BioGRID (Stark et al., 2011), IntAct (Aranda et al., 2010), MINT (Ceol et al., 2010), DIP (Salwinski et al., 2004), and DroID (Murali et al., 2011)), altogether comprising 65,754 interactions between 8,025 proteins. (ii) interolog binary PPI network predicted from experimentally identified binary PPIs for human, mouse, worm, and yeast; (iii) network of interolog protein complexes predicted from experimentally identified protein complexes for human, mouse, worm, and yeast compiled from the BioGrid, IntAct, MINT, DIP, and HPRD (Keshava Prasad et al., 2009) databases, with the caveat that not all proteins in a complex necessarily participate in binary interactions with each other; (iv) kinase-substrate network. There is evidence in MasterNet supporting 74 of our 1,983 Y2H PPIs.

Construction of *Drosophila* TF–TF PRS and RRSs

MasterNet (Freeze April 2015) was used as a reference for published PPIs. We constructed the PRS by selecting interactions published in at least two studies. In addition, we required that at least one of the methods used in these studies showed a binary interaction, in order to construct a PRS of direct PPIs. We individually examined and verified the evidence in the referenced literature supporting the interactions in the PRS. This yielded 41 high-confidence PPIs in the PRS. We constructed the RRSs by generating random sets of interactions from the same TF space, maintaining similar numbers of interactions and node degree distribution in each RRS as in our Y2H network, and removing all previously known interactions (i.e., those in MasterNet).

PBM assays

Protein expression

We generated N-terminal glutathione S-transferase (GST), HA, or FLAG fusion constructs of the DNA binding domain region or full-length TF clones by Gateway cloning into pDEST15, pT7CFE1-NHA (Thermo Fisher Scientific), or pT7CFE1-NFtag (Thermo Fisher Scientific) expression vectors; we Gateway-converted the latter two vectors. Sequences were full-length verified. *In vitro* transcription and translation (IVT) reactions were performed according to the manufacturer's protocol (PURExpress IVT Kit (NEB, E6800) or 1-Step Coupled Human IVT Kit (Thermo Fisher Scientific, 88881)). Western blots were used to estimate molar concentrations of all *in vitro* translated proteins by utilizing a dilution series of recombinant GST (Sigma, G5663), FLAG (Sigma, P7582), or multi-tag protein including HA (Abcam, ab5395) as standard proteins. The following primary antibodies were used: rabbit anti-GST polyclonal primary antibody (Sigma, G7781; used at 20 ng/ml); mouse Alexa488-conjugated anti-HA monoclonal antibody (Thermo Fisher Scientific, A-21287; used at 1 μ g/mL); mouse anti-FLAG M2-horse radish peroxidase (HRP)-conjugated antibody (Sigma, A8592; used at 1 μ g/mL). The following secondary antibodies were used: goat HRP-conjugated anti-rabbit IgG (Pierce, 31460; added at 5 ng/ml); goat HRP-conjugated anti-mouse IgG (Thermo Fisher Scientific, 32430; used at 5 ng/mL). Glycerol was added to a final concentration of 30% to the completed IVT reactions samples prior to storage at -80°C .

PBM experiments using universal arrays

We employed an “all 10-mer” universal array design in $8 \times 60\text{K}$, GSE format (Agilent Technologies; AMADID #030236). Double-stranding of oligonucleotide arrays and PBM experiments were performed following previously described experimental protocols (Berger and Bulyk, 2006, 2009; Berger et al., 2006). Briefly, the TF of interest was expressed with an epitope tag (GST, HA, or FLAG), applied to the double-stranded DNA array, and detected with fluorescently labeled antibody specific for the tag. Experimental conditions used for all PBM experiments, including TF concentrations and buffers, are provided in Table S5. The following Alexa Fluor 488-conjugated antibodies were used: anti-GST (Thermo Fisher Scientific, A-11131), anti-HA (Thermo Fisher Scientific, A-21287), and anti-FLAG (Cell Signaling, 5407).

PBM data analysis

Microarray data quantification and normalization were performed as described before (Berger and Bulyk, 2006, 2009; Berger et al., 2006) using the Universal PBM Analysis Suite. TF DNA binding specificities were derived using the Seed-and-Wobble algorithm (Berger and Bulyk, 2009). Success in motif derivation from universal PBM data was assessed according to seed 8-mer enrichment score (E-score) and obtaining at least five 8-mers with E-score at least 0.45 matching the derived motif. The E-score is a modified form of the Wilcoxon Mann-Whitney rank-based statistic and is robust to technical variation across arrays. Larger E-score values reflect higher specificity of a TF for a particular 8-mer. Motif sequence logos representing the derived DNA binding specificity position weight matrices (PWMs) were generated using enoLOGOS (Workman et al., 2005).

Motif co-occurrence analysis

Motif compilation and data processing

We obtained 307 DNA binding site motifs (Table S6) as PWMs from FlyFactorSurvey (FFS) via the MEME motif database download (Zhu et al., 2011) or from our newly generated PBM data. For FFS-derived PWMs, we matched each PWM to a TF or to both TFs in a heterodimer pair and separated out those TFs relevant to our analysis (Tables S2) for further consideration. FFS curates PWMs generated from a variety of experimental methods and collected from multiple sources. If there were multiple PWMs associated with a TF, we preferentially chose a single PWM from PBMs performed in this study, then from FFS (Zhu et al., 2011) with the following evidence codes, in decreasing order of priority: SOLEXA, FlyReg, SANGER, Cell, NAR, and NBT. If there were multiple PWMs available from a chosen source, we manually chose a representative(s) PWM for each TF. Special cases included isoforms and dimers. For isoforms, we chose the PWM representing the assayed TF isoform, where available; for dimers, we chose PWMs representing homodimers, where available. We also added two motifs not found in FFS nor assayed by PBM in this study, but needed for the ChIP analysis (see below): Stat92E and gcm were taken from CIS-BP (Weirauch et al., 2014) with IDs M2327 and M2560, respectively. We trimmed both the 5' and 3' ends of all chosen PWMs until two consecutive positions with information content greater than 0.5 were obtained, as previously described (Barrera et al., 2016).

Assembling and processing ChIP-chip/seq data

ChIP-chip/seq datasets were gathered from modENCODE and a search of GEO DataSets for dm3 ChIP-seq datasets. Altogether, we assembled a total of 57 datasets applicable to our analysis (Table S7). Peaks were taken directly from the published analyses. For each ChIP dataset, we defined two sets of euchromatic regions: the foreground (i.e., ChIP ‘bound’ regions, as defined by the authors of each ChIP dataset) and the background (i.e., genomically matched regions not found in the foreground). We took the top 500 peaks

of each set (as denoted by signal value) and trimmed them to 200 bp around the peak summit (i.e., peak summit \pm 100 bp). If summit information was not available, then we trimmed to 200 bp around the center of the peak (i.e., peak center \pm 100 bp). The liftOver tool (Hinrichs et al., 2006) was used to convert dm2 mappings to the dm3 genome, and any unmapped regions were discarded. To generate matched genomic background sequences, we used GENRE (Mariani et al., 2017) to match for promoter overlap, repeat overlap, and GC content specified for the dm3 genome and 200-bp regions.

Analysis of ChIP-chip/seq data

We analyzed publicly available *in vivo* TF genomic occupancy data to determine the extent of direct versus indirect TF–DNA interactions *in vivo*. Here, we filtered interactions in the Integrated Network (Table S2) for the following criteria: (a) the two interacting TFs exhibited overlapping expression patterns as defined by the modENCODE Temporal Expression Data (Graveley et al., 2011); (b) DNA binding specificity data were available for both interacting TFs (see above); (c) ChIP-chip/seq data were available for at least one of the interacting TFs (see above); (d) TF expression pattern overlapped with the time point of the ChIP-chip/seq data; and (e) the DNA binding specificity motifs of the interacting TFs were dissimilar (using Pearson correlation coefficient < 0.8 as a threshold as in (Kheradpour et al., 2007)) in order to distinguish which TF's motif was enriched. For the 333 pairs that were left, we scored the foreground and background regions using a model similar to GOMER (generalizable occupancy model of expression regulation) (Granek and Clarke, 2005) to compute the probability that a TF binds a DNA sequence. We assessed the statistical significance of the area under the receiver operating characteristic curve (AUROC) value for a particular motif in a particular ChIP dataset by using a Wilcoxon rank sum test comparing the scores for foreground versus background sequences. We considered motif enrichment to be significant at Benjamini-Hochberg false discovery rate (FDR) $q < 0.1$.

Network analysis

Network visualization

We imported our Y2H network and the Integrated Network into Cytoscape (version 3.4.0) (Shannon et al., 2003) for visualization.

Comparison with nematode and human TF–TF interactomes

We compared our *Drosophila* TF–TF interactome against similar interactomes in *C. elegans* and human (Ravasi et al., 2010; Reece-Hoyes et al., 2013). Interactions in the *C. elegans* network were restricted to those between TFs as it contained interactions with co-factors, based on a list published in the same study, using a custom Python script. The numbers of interacting genes and basic network statistics were retrieved using NetworkAnalyzer (Doncheva et al., 2012), as implemented in Cytoscape.

To determine whether the hub TFs in our network were similarly highly connected TFs in the *C. elegans* and human networks, we compared the degree distribution of hub TF orthologs and that of the same number of randomly selected TFs in the respective species. We retrieved orthologs for our hub genes in the respective species through FlyBase. Because multiple orthologs exist for some *Drosophila* hub TFs, to avoid skewing the degree distribution, we randomly chose one ortholog to represent each hub TF in each of 10,000 samplings. The distributions of median node degree from each sampling were compared between the hub and random TFs using the Wilcoxon rank sum test; $p < 0.001$ was our threshold for significance.

Functional annotation retrieval

GO term annotation

GO Term annotations were retrieved from AmiGO 2 (<http://amigo.geneontology.org/amigo/>) (Balsa-Canto et al., 2016).

Ortholog annotation

We obtained human orthologs of *Drosophila* genes using the DRSC Integrative Ortholog Prediction Tool (DIOPT) (Hu et al., 2011). Because each *Drosophila* gene can have multiple human orthologs, we chose the top-scoring ortholog for the analysis shown in Figure 4, based on the DIOPT score, or based on what has been used as the ortholog in the literature. Additionally, human, mouse, rat, and zebrafish orthologs of fly genes were retrieved using Ensembl BioMart (Kinsella et al., 2011).

Tissue expression information

Drosophila tissue expression information was retrieved from the BDGP (Hammonds et al., 2013). The BDGP dataset includes tissue and developmental stage range information (henceforth, “expression term”). When one TF had a “ubiquitous” expression term, if the other TF was expressed in any tissue during the same developmental stage range as the ubiquitous term, these TFs were considered co-expressed and the expression term for the specific tissue only was taken as the co-expression term. If both TFs had ubiquitous terms at the same developmental stage range, the lesser ubiquitous term (e.g., “faint ubiquitous” as opposed to “strong ubiquitous”) was taken as the co-expression term.

Disease annotation

Human disease annotations for genes were retrieved from the Human Gene Mutation Database (Stenson et al., 2003).

TF family annotation

TF families were assigned based on domain annotations retrieved from (Hens et al., 2011). Sequence-specific DNA binding domains were manually curated based on annotations in Pfam (El-Gebali et al., 2019). If a TF did not have at least one curated DNA binding domain, it was classified into the “other” TF family.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis of Y2H-based interactions

PPIs were called automatically by a modified version of the TIDY software which calculates a Z-score for a given interaction over multiple different analyzed images (see “Automated detection of interactions in Y2H screen” section under [Method Details](#) for detail).

Sensitivity and specificity of the Y2H assay

The sensitivity and specificity of our Y2H assay were calculated against the PRS and RRSs, respectively, as described in the [Results](#) section.

PBM data analysis

DNA binding specificities of PBM-assayed TFs are represented as E-scores for individual 8-mers. Details can be found in the “PBM data analysis” section under [Method Details](#).

Statistical analysis for motif enrichment analysis

Details of this analysis can be found in the “Analysis of ChIP-chip/seq data” section under [Method Details](#) and are reported in [Table 2](#).

DATA AND SOFTWARE AVAILABILITY

TF–TF PPI data resulting from our Y2H screen: [Table S2](#).

The Integrated *Drosophila* TF–TF PPI Network: [Table S2](#).

The catalog of TF pairs demonstrating direct and indirect DNA binding: [Table 2](#).

The accession number for the universal PBM data reported in this paper is [UniPROBE (<http://thebrain.bwh.harvard.edu/uniprobe/>): [SHO18A]].

Custom scripts and software were used for: automated Y2H PPI detection; processing Y2H data; extracting tissue expression information from BDGP; extracting TF family information; extracting MasterNet information; comparing the *Drosophila* interactome with *C. elegans* and human interactomes; hub protein analyses; and the construction of RRSs. All custom scripts and software used in this study are available upon request.